THE PHOTOSENSITIZING ACTIVITY OF CHLOROPHYLL IN COACERVATES

K. B. Serebrovskaya, et al.

NASA TT F-10,711

Translation of "Fotosensibiliziruyushchaya aktivnost' khlorofilla v koatservatakh".
Biofizika, Vol. 7, No. 1, pp. 34-41, 1962.

NATIONAL AERONAUTICS AND SPACE ADMINISTRATION
WASHINGTON D.C. FEBRUARY 1967

THE PHOTOSENSITIZING ACTIVITY OF CHLOROPHYLL IN COACERVATES

K. B. Serebrovskaya, V. B. Yevstigneyev, V. A. Gavrilova, A. I. Oparin
(Institute of Biochemistry imeni A.N. Bakh, Academy of Sciences USSR, Moscow)

Coacervates containing chlorophyll are derived from serum albumin and potassium oleate in 50% alcohol. Experiments with coacervates having a definite chlorophyll content showed that the photosensitizing activity in the coacervate, chlorophyll-containing drops is significantly higher than in the surrounding liquid, which also contains chlorophyll. This paper presents some considerations to explain this phenomenon.

According to modern ideas (Ref. 1), the formation of coacervates in the /34* hydrosphere was an important step in the origin of life. The characteristic feature of living matter is the constant interchange of substances and energy with the surrounding environment; in this sense, all living organisms belong to the so-called open systems. A stationary state in an open system is always supported by the use of energy which must come from the environment in the form of chemical or radiant energy (Ref. 2).

From this viewpoint, a very important role in the development of life can be attributed to coacervate systems, which include the porphyrins, which are capable of converting the energy of a ray of the Sun into energy of the system. Colored compounds of this type may, as has now been established (Ref. 3, 4), be abiogenetically synthesized.

The Dutch school of experimenters has demonstrated that coacervate drops may, on the one hand, adsorb dyes (Ref. 5) and, on the other, may be formed under the effect of dyes (Ref. 6). It should therefore be expected that it would be possible artificially to derive coacervates which contained chlorophyll.

It has recently been shown that chlorophyll is capable of sensitizing the reactions of oxidation and reduction in the adsorbed (Ref. 7) and aggregated state (Ref. 8). In these cases the sensitizing action of chlorophyll apparently unfolds on the boundary between the reinforced sensitizer-pigment molecules and the aqueous medium containing the electron donor and acceptor (hydrogen).

In the first approximation, chlorophyll adsorbates may be regarded as models approximating the natural state of the chlorophyll in the chloroplast granules, where this pigment is concentrated in the form of thin, possibly monomolecular layers on a proteolipide carrier (Ref. 9). It should therefore be expected that the chlorophyll contained in coacervates might also possess the capacity of photosensitivation. From the methodological viewpoint, it would be very interesting to discover a fact of this sort, and it would open paths to further investigations.

---

* Numbers in the margin indicate pagination in the original foreign text.

Production of Coacervates Containing Chlorophyll

When producing coacervates for the above-indicated simulation, we proceeded from the fact that the bulk of the chloroplast granule is composed of proteins bound with lipides. In our work we therefore utilized a proteolipide coacervate derived according to the Bungenberg-de-Jong method (Ref. 10). The liquid phase of this coacervate comprised 50% ethyl alcohol. Human serum albumin obtained from the Vaccine and Serum Institute imeni I. I. Mechnikov was used as the protein component of the coacervate. This protein was chosen /35 because its isoelectric point is close to that of the gelatin utilized by Bungenberg-de-Jong. The lipide component of the coacervate was oleic acid.

To maintain the necessary pH, we used potassium tetraborate which we obtained by order of the Institute of Pure Reagents.

The coacervate was produced as follows: 370 mg of oleic acid and 73 mg of KOH were dissolved in 10 cc of a 0.2% solution of $K_2B_4O_7$. 200 mg of protein was dissolved in the same quantity of 0.2% solution of tetraborate. These solutions were mixed and gave a so-called reserve solution, which was stored at 4°C in a refrigerator. To form the coacervate, 0.5 cc of 0.75M KCl was added to 1 cc of the reserve solution. When an equal volume of concentrated solution of chlorophyll in alcohol (E_{665} = 60) was added to this coacervate suspension, a coacervate suspension in 50% alcohol was formed, and thus a ternary coacervate of protein-lipide-chlorophyll composition was obtained. The results derived from studying the composition of the coacervate derived are shown in Table 1.

TABLE 1

BASIC COMPOSITION OF COACERVATE OF SERUM ALBUMIN, POTASSIUM OLEATE, AND CHLOROPHYLL IN A 50% STANDARD*

Substance	Phase Distribution of Substance, %				Percentage of Each Component of Total Drop Mass
	Equilibrium Liquid		Drops		
1	2	3	4	5	6
1. Protein	45	17.2	55	137.5	32
2. Lipide	30	11.5	70	175.0	63
3. Chlorophyll	16	6.1	84	210.0	5

* Computation of the figures in columns 3 and 5 started with data in columns 2 and 4 and the volume of coacervate drops comprising 10% of the whole suspension.

The following methods were applied to analyze coacervate composition. The protein was determined according to the method of Kjeldahl*. To determine

* T. L. Auerman and N. V. Vasil'yeva determined the protein and ascorbic acid.

the chlorophyll, 3 cc of coacervate suspension was centrifuged at 2000 rpm for 10 minutes, the supernatant was diluted five times, and its optical density at 665 millimicrons (E_{665}) was found on a SF-4 spectrophotometer. The coacervate residue was dissolved in 15 cc of alcohol, diluted 50 times, and the E_{665} was measured. We were unsuccessful in performing a quantitative extraction of the lipide from the residue and particularly from the equilibrium liquid, since residue and liquid also contain the second lyophobic substance -- chlorophyll. We therefore determined the lipide by the difference when we knew the dry weight of the residue and the content in protein and chlorophyll.

The table shows that the bulk of the chlorophyll is concentrated in drops. The protein and lipide are also noticeably concentrated in drops.

Within certain limits, the period of centrifuging exerted no effect on the completeness with which the coacervate drops were precipated. The equilibrium liquid in all cases contained practically no drops (inspection at a magnification of 900 times under the microscope) (Table 2).

TABLE 2

EFFECT OF CENTRIFUGING PERIOD ON PRECIPITATION OF COACERVATE DROPS

Centrifuging Period, min	5	10	15	20	25
Chlorophyll in Equilibrium Liquid, % of that used in experiment	17.4	16.3	16.3	15.4	14.1

It was interesting to note out how different concentrations of chlorophyll/36 in the alcoholic solution to be added affected drop formation, as well as whether chlorophyll concentration in the drops depended on chlorophyll content in this solution. Chlorophyll solutions with E_{665} values of 60, 30, 15, 7.5, 3.75, 1.87, and 0.94 were taken for experimentation. One and a half cc of each solution was added to 1.5 cc of coacervate suspension, and after drop formation this was centrifuged for 10 minutes. From the supernatant, which was a homogeneous liquid, we took 0.6 cc and added it to 6 cc of the dilution mixture. This mixture was prepared from 1 volume of the tetraborate used in the experiment, 0.5 volume of 0.75M KCl, and 1.5 volume of alcohol -- i.e., in the same ratio of osmotic factors in the medium as in coacervation. The dilute equilibrium liquid was subjected to spectrophotometry at 665 millimicrons.

The volume of coacervate drops in the suspension was determined by the following direct method. The coacervate suspensions were centrifuged at 2000 rpm for 10 minutes. The equilibrium liquid was carefully decanted from the residue, and its volume was measured. The equilibrium liquid volume in all chlorophyll concentrations which we studied proved to be 2.7 cc -- i.e., the drops comprised about 10% of the volume of the whole suspension.

From the literature, the following formula for determining coacervate residue volume by a refractometer is familiar (Ref. 11):

$$V_c = 100 \cdot \frac{n_m - n_e}{n_c - n_e},$$

where V_c is the coacervate layer volume, and n_c, n_e, and n_m, respectively, are the refractive indices of the coacervate layer, equilibrium liquid, and the system without coacervate.

We attempted to use this method for verifying the results of direct measurement, but we were unsuccessful in determining the coacervate layer's coefficient of refraction, which is required for calculations according to the formula. On the basis of the measurement results which we obtained, however, we were able to conclude that the main influence on the coacervate of serum albumin and potassium oleate is exerted by the alcohol, while changes in chlorophyll concentration have no perceptible effect on the volume of the coacervate layer. It may be assumed that if the refractive index of the equilibrium liquid does not change, then the data on the coacervate layer volume -- which we obtained by a coarser method -- nevertheless reflect the actual state of affairs.

TABLE 3

EFFECT OF CHLOROPHYLL SOLUTION CONCENTRATION ON DISTRIBUTION OF PIGMENT AMONG COACERVATE PHASES

No. in Order	E_{665}	Residue Volume	Equilibrium Liquid Volume	E_{665}		Concentration Ratio	E_x		Quantity Ratio	Dry Weight of Residue
				Of Drop	Of Equilibrium Liquid		Of Drop	Of Equilibrium Liquid		
1	2	3	4	5	6	7	8	9	10	11
1	30,00			4,26	193,05	45	11,08	77,24	7,0	0,0095
2	15,00			4,07	81,98	20	10,58	32,79	3,1	0,0066
3	7,50			2,74	35,70	13	7,12	14,28	2,0	0,0054
4	3,75	0,4	2,6	1,67	15,60	9	4,34	6,24	1,4	0,0039
5	1,87			0,92	7,13	8	2,39	2,85	1,2	0,0038
6	0,94			0,43	3,83	9	1,12	1,53	1,4	0,0033
7	0,47			0,26	1,58	6	0,68	0,63	0,9	0,0024

Table 3 summarizes the findings obtained on the influence of chlorophyll solution concentration, which is added during coacervate formation, upon the chlorophyll distribution between the equilibrium liquid and drops. Column 2 gives the optical density at 665 millimicrons after a twofold volume increase when the solutions are mixed.

Column 6 gives the measured values of the optimum density of the equilibrium liquid (e.l.) after centrifuging. Column 5 gives optical density of /37

drops computed from measurement of e.l. E_{665} and consideration of drop volume. Columns 8 and 9 show the amount of chlorophyll in conventional units (extinction per unit of volume is taken as the unit) in liquid and drops.

The data in Table 3 once more (see Table 1) indicate that there is a strong concentration of chlorophyll in the coacervate drops; in all cases the extinction coefficient of the drops is considerably higher than that of the equilibrium solution. The extinction ratio increases as concentration of the original solution becomes greater.

Pigment concentration in the drops is apparently primarily associated with increased concentration of the basic coacervate components -- protein and lipide -- which include the chlorophyll. However, as follows from Table 1, the concentration of these components in the drops goes up perceptibly less than does pigment concentration. The conditions for adsorption of chlorophyll on the protein and lipide are apparently generally more favorable in the drops than in the equilibrium liquid.

The reason why the dry weight of the drop residue (Table 3, column 11) in an identical volume noticeably decreases as concentration of the original chlorophyll solution diminishes is not entirely clear. It may be assumed that this involves, first, a reduction in the amount of chlorophyll entering the drops, and, secondly, the lesser hydration of the protein-lipide complex when there is a large amount of pigment.

It is as yet difficult to say with exactitude in what state the chlorophyll in the investigated coacervate system occurs. However, the presence of an appreciable amount of pigment in the equilibrium liquid, the red fluorescence of the drops and equilibrium liquid, and the results of microscopic observations foster the conclusion that the chlorophyll in this system is chiefly in a state of adsorption on the molecules of the protein-lipide complex -- in the same way as on detergents.

Because of decreased molecule hydration in coacervate formation, the conditions for chlorophyll adsorption become even more favorable, which leads to increased chlorophyll concentration in the drops.

When more concentrated chlorophyll solutions are used -- as, for example, in experiments 1 and 2 (Table 3) -- surface adsorption of chlorophyll on the molecule aggregates may apparently also occur. An indication of this is the fact that the chlorophyll passes into solution more readily in this case when washing with the equilibrium liquid (without chlorophyll).

Photochemical Experiments

To study the photosensitizing capacity of chlorophyll found in coacervates, we selected the reaction of reducing methyl red by ascorbic acid. These compounds do not interact in darkness. This reaction has been repeatedly employed in previous experiments (Ref. 7 - 9).

Methyl red and ascorbic acid were introduced into a coacervate suspension containing chlorophyll, and the mixture obtained was illuminated under anaerobic conditions through a red filter passing light absorbed only by the chlorophyll.

If the chlorophyll possesses photosensitizing activity, the methyl red should gradually lose color under illumination, as may be judged by the findings of spectrophotometry at 520 millimicrons.

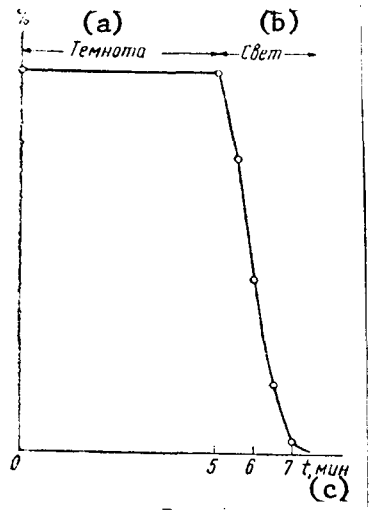

Figure 1

(a) - Darkness; (b) - Light;
(c) - t, min

Figure 1 gives the results of one of these experiments. It follows from this that the chlorophyll in the coacervate system does in fact -- like aggregated and adsorbed chlorophyll -- have the capacity to photosensitize oxidation and reduction reactions. /38

The coacervate system, however, is distinguished from the above-mentioned systems in that the pigment is contained in this case not only in the concentrated structures, but also in the surrounding liquid. Therefore, the question arises as to the relative photochemical role of the pigment situated in one phase or the other.

To resolve this question we centrifuged the equilibrium liquid from the coacervate drops, conducted the photochemical reaction with it, and compared the efficiency of this reaction with that when the unseparated coacervate system was used.

From the previous experiments, it had become clear that it was necessary to conduct similar experiments with coacervates containing a small quantity of chlorophyll, because the high chlorophyll concentration in the drops caused severe screening and, as a result, reduced the efficiency of the action of the light absorbed. In experiments with concentrated solutions (e.g., as at the top of Table 3), removal of the coacervate residue by centrifuging not only did not reduce, but even accelerated, the speed of the photosensitizing reaction.

Figure 2 gives the results of comparing the course of the methyl-red decolorizing reaction when using coacervates obtained by employing very dilute solutions of chlorophyll in alcohol (1 -- equilibrium liquid, 2 -- suspension). We may see that in these cases, reaction efficiency is perceptibly higher in the presence of drops.

It should be mentioned that, in order to conduct spectrophotometric measurements, the photochemical experiments were in every case carried out with

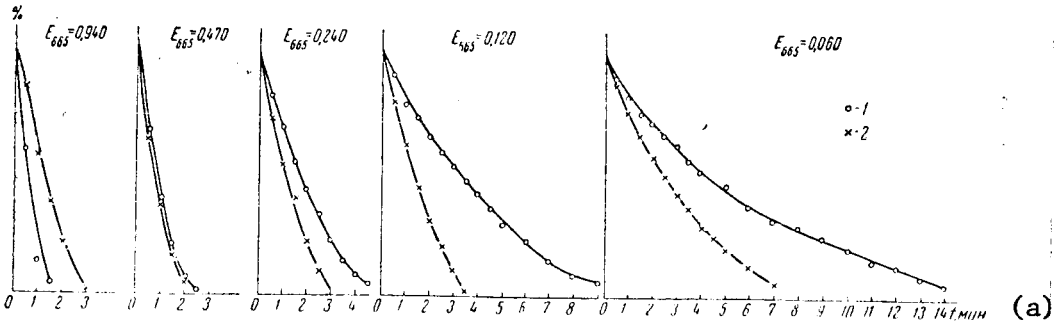

Figure 2

(a) - t, min

elevenfold dilution of the original suspension. The volume of the drops in these experiments, therefore, did not exceed 1% of the total volume. In spite of this small volume, the presence of drops in the solution exerted a clearly apparent accelerating effect on the course of the reaction.

In Table 4 we have attempted to make a numerical comparison of the efficiency of the sensitizing action of the equilibrium liquid and of the drops and have scaled this efficiency per unit of volume. /39

TABLE 4

SENSITIZING EFFECT IN SUSPENSION AND EQUILIBRIUM LIQUID

Quantity of Chlorophyll	V, in Suspension	V_1, in Equilibrium Liquid	$V-V_1$ in Drops	Rate per Unit of Volume		Rate Ratio per Volumetric Unit
				of equilibrium	of drop	
1	2	3	4	5	6	7
0,47	36	33	3	8,25	75,00	9
0,24	46	31	15	7,75	375,00	48
0,12	38	21	17	5,25	425,00	81
0,06	23	17	6	4,25	150,00	35

As the measure of the reaction rate, we conventionally chose the quantity of methyl-red decolored in the first minute of illumination expressed as a percentage of its original quantity, which was almost the same in all experiments (columns 2 and 3).

The portion of decolored methyl red per drop (column 4) was obtained as the difference between the data in columns 2 and 3.

The figures of columns 5 and 6, derived by dividing the figures in columns 3 and 4 by the volumes of liquid and residue (4 cc and 0.04 cc), respectively, show the amount of methyl-red which would be decolored in the presence of a

unit volume of the equilibrium liquid (column 5) and of the drops (column 6). These figures clearly indicate the substantially greater efficiency of the sensitizing action of the coacervate drops in comparison to the similar influence of the equilibrium liquid.

It is natural that the reason for this fact is the appreciably greater chlorophyll concentration in the drops. However, a simple calculation shows that the ratio of chlorophyll concentration in drops and liquid is after all perceptibly less than the corresponding ratio of the reaction rates.

If, in fact, we take as an example the experiment with an initial chlorophyll optical density of $E_{665} = 0.12$, then the data in columns 5 and 6 of Table 3 (taking into account the elevenfold dilution of the equilibrium liquid) indicate that the concentration of chlorophyll in the drops -- which has remained unchanged during dilution of the suspension -- exceeds its concentration in the equilibrium liquid by a factor of 2.5 x 11 = about 28, while the reaction is 81 times more intense (column 7, Table 4).

From this it follows that the intensity of the photosensitizing action of the chlorophyll in the drops at the given initial concentration is 2 to 2.5 times higher than in the equilibrium liquid.

A similar picture is also derived in the case of even more dilute solutions. For a case with $E_{665} = 0.06$, there was a rise in chlorophyll concentration of 1.7 x 11 = 19 times, while the reaction rate rose by a factor of 35.

In the coacervate drops, conditions are therefore apparently established which favor a certain intensification of the photosensitizing activity of chlorophyll. The cause of this may be the change in the state of the chlorophyll itself, which in our case is unlikely. At the same time there may be an improvement in the conditions under which the reaction takes its course, an improvement which depends on the other reaction components -- hydrogen donor and acceptor, in particular -- because of their partial adsorption.

In order to derive data on the effect exerted by coacervate drop formation/40 on the phase distribution of ascorbic acid and methyl-red, we determined the content of these substances in the whole system and in the equilibrium liquid after centrifuging. Coacervate solutions diluted eleven times were utilized for the experiments. The methyl-red was spectrophotometrically determined at 520 millimicrons. The ascorbic acid was found by Fujita and Ebihara's method (Ref. 12) with 2,6-dichlorophenylindophenol.

The findings listed in Table 5 indicate that the ascorbic acid is apparently uniformly distributed through the whole system, while the methyl-red is concentrated in the drops, but to an appreciably lesser degree than chlorophyll. If, however, we take the small volume of the drops into consideration, then the lesser extinction of the methyl-red in the equilibrium liquid may after all signify a substantial concentration of dye in the drops, and this may be conducive to development of the photosensitized reaction.

TABLE 5

DISTRIBUTION OF DYE AND ASCORBIC ACID BETWEEN COACERVATE PHASES

Mixture Name	Ascorbic Acid, cc	Phase Dye, E_{520}
1. Coacervate	36.6	0.422
2. Equilibrium liquid	36.6	0.395

The experiments described therefore indicate that the formation of coacervates apparently favors the photosensitizing activity of the pigment, in comparison to the system with uniform distribution of the sensitizer.

The coacervate drops may apparently be regarded as circulating systems in which the electron donor and acceptor (hydrogen) are continually entering and from which the reaction products leave

$$S \rightarrow \boxed{A \rightarrow B} \rightarrow Z$$

As a result of the great intensity of the sensitized reduction of methyl-red by ascorbic acid within the drops, the concentration of these substances in the drops in fact rapidly becomes less than that in the equilibrium liquid. As a consequence there is intensive diffusion of these reagents from the external liquid within the drops. At the same time the great concentration of reaction products within the drops causes them to diffuse into the surrounding solution.

We should like to regard the chlorophyll-containing, illuminated coacervate drops as one of the most primitive forms of metabolism, which takes place under the influence of absorbed light energy.

Conclusions

1. Coacervates containing chlorophyll are derived from serum albumin and potassium oleate in 50% alcohol. Pigment concentration in the coacervate drops proved to be substantially higher than in the equilibrium liquid. From a series of observations, it may be assumed that the chlorophyll in the given system is chiefly found in the adsorbed state on the molecules of the proteolipide complex, which acts as a detergent.

2. The chlorophyll-containing coacervate suspension has a photosensitizing influence upon the oxidation-reduction reaction between methyl-red and ascorbic acid, which do not react with each other in darkness.

Experiments with coacervates having a definite chlorophyll content showed that the photosensitizing activity in the coacervate, chlorophyll-containing

drops is significantly higher than in the surrounding liquid, which also contains chlorophyll. This paper presents some considerations to explain this phenomenon.

Received
January 21, 1961

REFERENCES

1. Oparin, A. I. Proiskhozhdeniye zhizni na Zemle (The Origin of Life on Earth). Moscow, 1957.
2. Pasynskiy, A. G., Blokhina, V. P. Genesis of Life on Earth. Trudy Mezhdunarodnogo Simpoziuma, 19-24 Avgusta, 1957 g., Moskva (Transactions of the International Symposium 19-24 August, 1957). Izdatel'stvo AN SSSR, Moscow, 1959.
3. Scott, J. Biochem. J., 62, 6, 1956.
4. Triebs, A. Conférences et reports du 3-ème congrès internat. de biochem., Bruxelles, 1-6 août 1955 (Lectures and Reports at the Third International Biochemical Congress, Brussels, 1-6 August, 1955), p. 207, Liege, 1956.
5. Bungenberg-de-Jong, H. G., Bank, O., Hoskam, E. G. Protoplasma, 34, 30-54, 1940.
6. Bungenberg-de-Jong. Chem. Abstr., 33, 915, 1939.
7. Yevstigneyev, V. B., Gavrilova, V. A. Doklady Akademii Nauk SSSR, 126, 410, 1959.
8. Yevstigneyev, V. B., Gavrilova, V. A. Biofizika, 4, 641, 1959.
9. Rabinovich, E. Fotosintez, 3, Moscow, 1959.
10. Bungenberg-de-Jong, H. G., Mallec, C. Proc. Konink. Nederl. acad. wet. B., 55, 360, 1952.
11. Booiy, H. L., Lycklama, J. C., Vogelsang, C. J. Proc. Koninc1. nederl. acad. wet., 52, 9, 1949.
12. Fujita, A., Ebichara, T. Biochemische Zeitung, 290, 172, 1938.

Scientific Translation Service
4849 Tocaloma Lane
La Canada, California